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Structure and biodegradation mechanism of milled *B.mori* silk particles

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Abstract

Silk particles with a volume median $d(0.5)$ of about 6 μm were prepared using a chemical free and efficient wet milling-spray drying process. Milling reduced the intermolecular stacking forces within the β -sheet crystallites without changing the intramolecular binding energy of the β -sheets. The rough morphology and the ultrafine size of the particles were responsible for significant surface modulated protease XIV degradation, about a three-fold increase compared to silk fibres. Fracture of brittle and porous enzyme hydrolysed particles produced a 72% fall in $d(0.5)$ within the *in-vitro* experimental conditions. Of note, upon biodegradation, the thermal degradation temperature of silk increased, which was attributed to the formation of tight aggregates by the hydrolysed residual fibroin macromolecules. A model of the biodegradation mechanism of silk particles was developed based on these data. The model explains the process of disintegration of β -sheets within the microstructure, supported by quantitative secondary structural analysis and microscopic images. This study is useful to engineer silk particles for targeted biomedical applications and also in understating structural remodeling of debris if generated from silk-based implants.

Keywords

silk; particles; enzyme; structure change; biodegradation

Introduction

Silk fibroin-based biomaterials have been developed for a broad range of biomedical and biotechnological applications leading to the transition of silk from traditional textile fibres to advanced materials [1-5]. Among these, micro and nano sized silk particles have shown promising results as resorbable vehicles for drugs and growth factors for diagnostic and tissue engineering applications [4, 6, 7]. Particles have been used also as fillers in composite scaffolds [8, 9]. One of the critical factors for success of a resorbable biomaterial in clinical applications is the process of biodegradation. In the case of particles, degradation kinetics may affect their functions as carriers of therapeutic or diagnostic agents. In tissue engineering, biodegradation rate of the scaffold material is critical as it should match the rate of tissue regeneration. Degradation rate is also important for controlling cellular metabolism and differentiation [10].

Kinetics and mechanisms of degradation of silk have been studied using *in-vitro* models, but mostly restricted to silk films, fibres and foams. Numata *et al.* reported that 5 nm thick crystal like structures could be fragmented by protease XIV to about 2 nm thick

nanofilaments [11]. A decrease in β -sheet content from about 50% to about 30% was found as a result of this enzymatic hydrolysis. However despite the higher expected degradation rate of the non β -sheet phases, the proportional increase of such fractions remained elusive [11]. Lu *et al.* reported faster degradation of silk films containing a high silk I phase structure (non β -sheet) than those with higher silk II phase (β -sheet) [12]. On the other hand stretched stabilised films with substantial β sheet content degraded rapidly [13]. Such results demonstrate that the degradation of silk is not always directly proportional to β -sheet content and may depend on architecture and distribution of different conformational phases in the fine structure of silk.

The structure of a silk material depends largely on the manufacturing method. Like most silk-based biomaterials, silk particles are usually prepared through a bottom up approach via dissolution and then regeneration of fibre protein [5]. However, in regenerated silk, the original microstructure of the fibre is difficult to reform [14, 15]. In addition, silk regeneration often relies on the use of chemicals such as lithium salts and can utilize significant quantities of water. On the other hand, milling silk fibres is an alternate and attractive approach to develop fine silk particles, avoiding chemicals and utilizing less water [16]. Unlike the bottom up approach, the top down process utilizes only mechanical energy to reduce particle size.

The objective of this work was to gain insight into the morphology and fine structure of milled silk particles and to correlate their secondary structure with enzymatic degradation. Protease XIV was used due to its high activity towards silk β -sheet structure [11, 17]. Insight into the structure and degradation relationships of silk will assist in tuning its degradation behaviour through optimisation of the milling process and thereby will further assist in developing targeted biomedical applications with milled silk particles.

In addition to degradation kinetics and structure-degradation relationships, this study examined the structures of enzyme hydrolysed silk. The residues of biodegradation can be the major causes of inflammatory reactions impacting the success rate of a biomaterial. It is known that conformation of certain structural proteins such as prion protein, PrP, contributes to neuro-generative diseases [18]. According to some preliminary studies, silk materials containing β sheets have not shown cytotoxicity [11]. However, conformations of degraded products and associated influence on cytotoxicity have not been well investigated. As part of such investigations, this study examined secondary structures of *in vitro* degraded silk particles with the aim to understand structural remodeling during enzyme action. It is hoped that the study will be useful to predict the behaviour of particulate debris generated from various forms of silk materials.

Materials and Methods

Materials

Stiffled *Bombyx mori* silk cocoons were sourced from northeast India (details?). All reagents, if not stated otherwise, were purchased from Sigma Aldrich Australia Ltd.

Silk degumming

The silk cocoons were cut to remove pupae. To remove sericin, degumming was performed in a laboratory dyeing machine (Thies, USA) using 2 g/L sodium carbonate and 0.6 g/L sodium dodecyl sulphate (Sigma-Aldrich, Australia) at 100°C for 20 min with a material mass (g) to liquor volume (mL) ratio of 1:25. After degumming, the cocoons were washed thoroughly with warm distilled water (dH₂O) followed by cold dH₂O.

Powder Production

Silk powder was produced using the method reported earlier [16]. Briefly, degummed silk fibres were chopped in a cutter mill (Pulverisette 19 from Fritsch GmbH, Germany) fitted with a 1 mm grid and operating at 2888 rpm. Chopped snippets were wet milled for 6 hours in a stirred media mill (1S Attritor from Union Process, USA). Stirrer speed was set at 280 rpm and 20 kg yttrium doped zirconium oxide balls of 5 mm diameter were used as the milling media. Batch size was 200 g of snippets and 2L of DI water. Cooling water (approximately 18°C) was circulated through the vessel jacket during milling. After wet milling, the slurry was spray dried (B-290 from Buchi Labortechnik AG, Switzerland) to produce silk powder using the following conditions: inlet temperature, 130°C; pump setting, 25% (18–20 mL/min); and aspirator setting, 100% (42.5 m³/h).

Enzymatic degradation

The degradation of the silk particles was evaluated using protease XIV from *Streptomyces Griseus* (Sigma–Aldrich) with an activity of 3.5 U/mL. Powder specimens of about 100 mg were conditioned at 20±2°C and 65±2% relative humidity for 24 hours and weighed. Specimens were then treated with 10 mL of enzyme solution prepared using 0.1M phosphate PBS (pH 7.4) and were agitated inside the incubator at 37°C. Enzyme concentration was 1 mg/mL in the slow degradation experiment in which the solution was replaced with fresh solution every 2 days to maintain the enzyme activity. In the rapid degradation experiment, 2 mg/mL solution was used and the solution was replaced every day. Specimens were collected at 3 hr, 2, 16, 30 and 56 days in case of slow degradation and 3, 6 hr, 1, 3, 6 and 12 days in the case of rapid degradation tests. The number of specimens was 3 at each time point. At the end of degradation, powders were treated in 15 mL 2% acetic acid solution for 30 min at room temperature followed by rinsing in 15 mL dH₂O at room temperature. Rinsing was repeated 5 times. During change of enzyme solution and rinsing, samples were centrifuged at 4,800 rpm for 15 minutes and there was no evidence of particles in the discarded supernatants. Control samples were treated only in PBS solution. Replacement of PBS solution, and rinsing cycles of the control specimens were maintained similar to the enzyme treated samples. At the end of rinsing, samples were put in liquid N₂ and lyophilized (LABCONCO- FreeZone). Dry particles were conditioned again at 20±2°C and 65±2% relative humidity for 24 hours and weighed to determine the weight loss.

Particle size distribution

Prior to freeze drying, 20 mL of enzyme treated and rinsed particle suspension in water was collected for measurement of size distribution. To break the aggregates formed from repeated centrifuge during change of enzyme solutions and rinsing, the suspensions were sonicated for 30 seconds at 70% amplitude. Particle size distribution was measured using a Mastersizer 2000 (Malvern Instruments, UK) fitted with Hydro 2000S. The dispersion medium was DI water. Data analysis was performed by the Malvern software using the following material parameters: refractive index of silk 1.561, absorbency of silk 0.1. Each sample was measured four times. All results were presented according to a volume-based particle size distribution.

Scanning electron microscopy

Morphology of the particles was observed under a scanning electron microscope (Carl Zeiss AG - SUPRA 55VP) at 1-2 kV accelerated voltage and 1.5-4.5 mm working distance. Prior to SEM imaging, particles were made conductive by approximately 1 nm thick evaporated carbon coating.

FTIR analysis

Infrared spectra of desiccated silk particles were recorded with an attenuated total reflectance (ATR) Fourier Transform Infrared (FTIR) spectrophotometer (Vertex 70) (Bruker Biosciences Pty Ltd, Australia). Each spectrum was obtained in absorbance mode in the range of 4000-600 cm^{-1} . To measure different conformations, average spectrum in the amide I mode was deconvoluted and curve fitted (1595-1705 cm^{-1}) using OPUS 5.5 software, adapting the procedure used earlier after slight modifications [19]. Briefly, deconvolution was carried out adapting a Lorentzian model using a bandwidth of 25 cm^{-1} and a noise reduction factor of 0.3. Baseline was corrected before and after deconvolution. Curve fitting was done using the Gaussian model by the autofit programme using initially Local least square followed by Levenberg-Marquardt algorithms. Fixed band widths (5.015) around the following positions were initially selected based on FSD spectra and known conformational positions of amide I regions: 1619 cm^{-1} (intermolecular β sheet), 1627 cm^{-1} (intramolecular β sheet), 1647 cm^{-1} (random coil), 1666 cm^{-1} (β turn), 1680 cm^{-1} (β turn), 1696 cm^{-1} (tight β turn) and 1700 cm^{-1} (strong β turn). Finally, each individual spectrum was area normalised to obtain percentage conformations within the amide I region. The procedure was repeated four times with slight changes in positions of initial selection for each spectrum.

Thermal properties

Differential scanning calorimetry (DSC) measurements were made in a TA Instrument Q100 DSC (TA Instruments, New Castle, DE) under a dry nitrogen gas flow and equipped with a refrigerated cooling system. Desiccated specimens of about 5 mg were used for the measurements and were encapsulated in aluminium pans. The samples were heated at 5°C min^{-1} from -30°C to 350°C. Readings were analysed using NETZSCH Proteus-Thermal Analysis Software.

X-ray scattering analysis

Wide angle X-ray scattering (WAXS) was performed on a diffractometer (PANalytical-Xpert PRO) with Cu K α radiation ($\lambda = 0.154$ nm) operated at 40 kV and 30 mA. Scanning rate was 0.01°/s. A low noise single crystal Silicon specimen holder was used to avoid any interference of background scattering. Spectra were analysed using the Xpert High Score Plus software.

Results

Change in size and mass of silk fibres and particles

B.mori silk fibres were chopped and wet milled in an attritor for 6 hrs to get a stable suspension of fine particles in water with a volume $d(0.5)$ of 6.4 μm . The suspension was then spray dried to generate silk powders. There was no change in particle size during spray drying. Despite milling under circulating chilled water to avoid thermal degradation during milling, mechanical energy in the powdering process caused some decrease in molecular weight of fibroin (see Fig.S1). Milled powder was treated in a PBS solution with or without enzyme. As illustrated in Fig.1 and Fig.2, 12 days exposure without enzyme (pH 7.4, 37°C) caused no change in volume based $d(0.5)$ and mass of the particles. The results confirm that the despite much larger surface area and decrease in molecular weight compared to the fibres, the mechanically produced ultrafine silk particles were insoluble in PBS solution.

The changes in particle size distribution and mass loss as a result of protease XIV hydrolysis were monitored at different time points. Concentrations of protease XIV were 1 mg/mL and 2 mg/mL and corresponding experiments were termed as slow and rapid degradation, respectively. To maintain activity, enzyme solutions were replaced every 2 days in the slow

degradation studies and every day in the case of rapid studies. As illustrated in Fig.2, total mass loss and rate of mass loss of the particles were significantly higher than the fibres. In the slow degradation study, particle mass dropped rapidly to less than 60% in about 6 days. Degradation rate was slower thereafter and the remaining mass was about 40% at the end of 8 weeks. In the case of fibres, there was no indication of degradation during the first 2 weeks. Mass loss was about 20% in 30 days and reduction thereafter to 8 weeks was only marginal ($p > .05$, student t test). High rate of degradation of powders could be further accelerated in the rapid tests. More than 50% mass was removed by protease XIV within 3 days though the degradation slowed down significantly thereafter. Concentration as well as enzyme replacement rate influence the kinetics of degradation as protease is known to lose activity after about 1 day [20]. Mass loss profile in the case of fibres is similar to the results reported by Horan *et al.* though the scale of degradation is different [20]. They reported about 50% mass reduction for *B.mori* silk fibres in about 6 weeks when exposed to a 1 mg/mL protease XIV solution. A relatively higher degradation rate in the previous study can be attributed to more frequent change of enzyme and use of higher substrate to enzyme solution ratio (1:70 against 1:10 used in this study), which are known to be important determinants of degradation rate [21].

Volume based particle size distributions of *B. mori* silk particles at different degradation time points are presented in Fig.3. Median diameters of particles are shown in Fig.1. The $d(0.5)$ value dropped from 6.4 μm to 1.8 μm at the end of 12 d indicating a reduction in equivalent microsphere diameter by about 72%. A substantial shift of the left tail of particle distribution, particularly during the later part of degradation, suggests generation of a large number of finer particles. A surface erosion mechanism was proposed to explain the degradation of silk materials by protease XIV[20]. Considering such a surface erosion process, a mass loss of 60%, occurred in the case of 12 d degraded particles (Fig.2), would result a reduction in sphere diameter by 23% only. Silk particles used in this study are not solid spheres but highly porous and the enzyme degradation could progressively create larger pores and subsequent fracture into finer particles (Fig. 4). We hypothesize that substantial fragmentation of the particles occurred during the hydrolysis as a result of enzyme penetration into the pores. Such infiltration of enzyme resulted in particle fragmentation and generation of fine particles and hence higher reduction in particle size than that would occur from only surface erosion. A volume based distribution was used to measure particle size in this study. Hence any large number of ultrafine insoluble debris from surface erosion representing a small fraction of mass alone can not shift the size distribution substantially. Hence the shift in distribution was primarily due to particle fracture. Fig.5 illustrates the images of silk fibres before and after enzyme treatment. The images provide evidence of surface erosion of silk fibres and severe fracture. Silk becomes excessively brittle due to enzyme treatment allowing fracture to take place even without any application of force. So the increase in brittleness assisted fracture of particles during protease XIV treatment. Mass of the particles decreased only marginally towards the end of degradation tests (*i.e.* from 3 d to 12 d time points). Thus fragmentation of particles was responsible at that stage for the reduction of particle size. A mild ultrasonication was used to break aggregates before measurement of particle size distribution. There was no difference in particle size between milled slurry and 12 d PBS only treated samples. Though sonication could fracture brittle particles to some extent, we suggest enzyme hydrolysis was primarily responsible for particle fracture and size reduction.

Change in secondary structure

FTIR amide I (1600-1700 cm^{-1}) region reflects C=O vibration in the protein backbone. The band positions in the region change depending on the backbone conformation and therefore can be used to determine molecular conformation. The 1616-1637 cm^{-1} region represents β -

sheet [19, 22] and the 1638–1663 cm^{-1} region is usually attributed to amorphous protein. Within the amorphous region, there is a general agreement to use the 1638–1655 cm^{-1} for assigning random coil and the remaining region to helical conformation. The 1665–1690 cm^{-1} is assigned to β -turn [19, 22] whereas a strong band in the region 1691–1700 cm^{-1} is often used for β -sheet [19]. However, using real time FTIR during silk crystallisation, Chen *et al.* proposed the band in the region 1691–1700 cm^{-1} did not appear due to β -sheet; instead they assigned this to β -turns possibly together with a component derived directly from β -sheet [23]. Considering the positive correlation of the band in the region 1691–1700 cm^{-1} with crystal formation in silk [23], we have distinguished it from other β -turn and define it as tight β -turns. Formation of these tight β -turns is reflected prominently in FTIR results of enzyme treated samples, but they do not enhance X-ray reflection (to be discussed subsequently) as opposed to β -sheets that add to X-ray peaks.

In Fig.6, amide I absorptions normalised using OPUS5.5 software are shown for degummed *B.mori* silk fibre, 30 min, and 12 d enzyme treated powders and the powder treated for 12 d in PBS. There is no difference in the patterns of powders after PBS treatment and hence only 12 d PBS treated plot is presented in Fig.6. There is a noticeable shift in the β -sheet peak from around 1619 cm^{-1} to around 1625 cm^{-1} after milling. On the other hand, protease XIV hydrolysis has not changed the β -sheet position from around 1625 cm^{-1} . As illustrated by the arrows, there have been small but continuous shifts (decrease) in the amorphous region during enzyme treatment. The height and position of the tight β -turn peak around 1697 cm^{-1} remain unchanged but due to the depression in the band in the amorphous region, sharpness of the peak has increased after biodegradation.

For improved resolution and estimation of different conformations, the broad amide I band was Fourier self deconvoluted (FSD) and curve fitted to yield a set of bands from which the conformation could be determined. Second derivatives of the original FTIR spectra were used to select band positions that were then allowed to shift using autofit option in OPUS5.5. Based on surveyed literature, we earlier reported on dividing the β -sheet region of 1616–1637 cm^{-1} into intermolecular (1616–1627 cm^{-1}) and intramolecular (1616–1627 cm^{-1}) sub-zones [19]. Therefore the initial band positions around 1619 cm^{-1} and 1630 cm^{-1} have been selected to assign intermolecular and intramolecular β -sheet fractions, respectively. Examples of curve fitted FSD for fibre, PBS only and 12 d protease XIV treated powders are presented in Fig. 7. The solid curve is the resultant spectrum, and the dotted curves are the individual deconvoluted peaks. The percentage conformations derived from area normalized data of fibre and powders at different time points of protease XIV treatment are presented in Fig.8. Standard errors from minor changes applied in band positions during curve fittings are very small and hence are not visible in Fig.8.

Milling caused a substantial fall in β -sheet (intermolecular + intramolecular) from 57% to 44% and an associated increase in random coil from 17% to 28%. The reduction in β -sheet can be attributed to decrease in intermolecular β -sheet content from 33% to 12% despite partial offset by some increase in the intramolecular fraction. Reduction in intermolecular β -sheet fraction in powders indicates that milling forces created defects in stacked β -sheets. β -sheet (intermolecular+intramolecular) in powder increased marginally from 44% to 48% at the end of 12 d ($p < 0.05$, student t test) biodegradation. Enzyme action also caused noticeable increase in tight turn (from 11% in PBS control powder to 14% in 12 d enzyme treated powder). The combined rise in β -sheet and tight β -turn is matched by an equivalent fall in random coil conformation, which dropped from 28% (PBS control) to 20% (12 d enzyme treated powders). β -turn fraction remained nearly unchanged (not shown in the Fig.8). The proportional change in amorphous contents, however, does not match the extensive mass loss (Fig.2), indicating that protease XIV did not remove proteins in the amorphous domain alone. Interestingly, proportional change in conformation was pronounced after 3 d when

mass loss was slowed down significantly. We therefore propose that during the initial phase of degradation, enzyme could penetrate into the amorphous as well as open β -sheet crystal defect zones removing both fractions nearly at a similar scale. The enzyme could not act on more compact β -sheet crystals or tight β -turn aggregates and primarily removed accessible amorphous domains slowing down the mass loss and increasing the β -sheet and β -turn fractions. Short chains created during enzymatic hydrolysis formed hooks and turns and produced tight aggregates and thus increased tight β -turn fractions.

Fig.9 presents normalised X-ray diffraction spectra of fibre and powder with or without protease XIV. Broad nature of the peaks come from the diffuse nature of reflections arising from the small size of crystallites superimposed by reflection from the amorphous halo[24]. Amorphous halo is due to the short range order related to chain-chain correlation constrained by interchain hydrogen bonding and the centre of the halo is close to the (210) peak position from silk crystals. Thus the silk WAXS peak for the β -crystals is fairly broad [25]. The peak centred around 20.3° is composed of two strong peaks at 18.92° and 20.80° from (200) and (210) crystal planes respectively [24]. Both these planes lie in the direction normal to crystal forming backbone GAGAGS sequence [24, 26]. (200) plane diffraction in particular is due to regular stacking of β -sheets. Therefore significant reduction in intensity and broadening of these peaks, as seen after milling, indicates increasing disorder between β -sheets in powders. This happens because the milling forces can destabilise the weak inter-sheet van der Waal stacking forces in silk crystallites. The apparent shift of the centre of the crystal peak from 20.5° to about 20.3° is also due to the increased intermolecular sheet packing distance due to higher side chain mobility caused by mechanical energy. The results support reduction in FTIR intermolecular β -sheet reflection and provide further evidence on destabilisation of β -sheet stacking as a result of silk milling.

A further reduction in peak intensity in 12 d protease XIV treated powder compared to PBS treated powder indicates that enzyme hydrolysis could reduce silk crystallinity. Such a change was not reflected in the FTIR results because any change in inter-molecular β -sheet was more than offset by decrease in amorphous contents due to enzymatic hydrolysis. A random short range order due to interaction of short chains is known to produce a halo with centre close to the 210 plane [24, 25]. A slight shift of the peak centre towards a higher angle (20.3° to 20.7°) reflects a decrease of the 200 plane component and an increase in short range order due to chain rupture and short chain aggregation.

Li *et al.* reported reduction in WAXS peaks for β -sheet and development of silk I crystal structure in protease XIV for *B.mori* films[17]. Silk I structure was proposed based on peaks at 11.7° , 19.7° , and 24.7° corresponding to 7.5Å, 4.5Å, and 3.6Å crystal spacing respectively[17, 27]. Protease XIV is not completely soluble in PBS at pH 7.4 and precipitates are mixed with the powder. Therefore a mild acid wash is needed to remove such residues from the silk powder (Fig.S2). We found that after treatment at pH 7.4, 37°C , protease XIV residual crystals show WAXS peaks reflecting similar crystal spacing assigned to silk I (Fig.S3). These crystal peaks particularly those at 11.7° and 20.8° disappears after the acetic acid wash providing clear evidence against converting β -sheet (silk II) to silk I structure by protease XIV treatment.

Change in thermal properties

Response to temperature by polymers including structural proteins varies depending on their molecular conformation and secondary structure. DSC studies were conducted on silk before and after milling and enzyme treatment (Fig. 10).

The endothermal peak at around 65°C is due to bound water and referred to as $T_g(1)$ [12]. In the case of fibre, it is at 63.5°C . The peak increases to 69.7°C after milling into powder

indicating a stronger interaction of bound water and silk powder. This stronger interaction is likely because of reduced intermolecular β -sheet providing access to moisture binding sites and faster kinetics of moisture molecules. $T_g(1)$ in powders decreases with the enzyme treatment, dropping down to the level of fibre after 12 d treatment. The decrease suggests reduction in moisture binding amorphous regions as confirmed by FTIR results.

The second endotherm starts above 250°C. Martel *et al.* confirmed that amorphous silk degrades at a temperature in which silk crystallites remain intact [25]. At a higher temperature, thermal energy delaminates β -sheets in the crystallisation region followed by their degradation [25]. Overlapping of disintegration and degradation of amorphous and crystalline regions is responsible for a broad peak and the stages can not be separated. The endothermic peak for both fibres and powders commenced at the same temperature (Fig.10). However the peak is sharper and degradation temperature lower in powder (284°C) compared to fibres (293°C). The crystal structure in powder is considerably opened up by the milling forces and hence a lower thermal energy is required for their disintegration whereas the compact crystallites in fibre require more thermal energy resulting in a higher degradation temperature. Wide distribution of crystallite size [24] and a combination of large amorphous region (lower degradation temperature) and stronger crystals in fibres (higher degradation temperature) are responsible for a wider endothermic peak in fibres. In the case of powder, the degradation happens quickly due to defects in crystallites, which result in a sharper endothermic peak.

It is shown in Fig. 10 that the degradation temperature of powder slowly increases after enzyme treatment. Onset of degradation at a higher temperature in enzyme treated powder is due to reduced amorphous phase. Progressive reduction of amorphous phase during enzyme treatment is suggested by FTIR results. Enzyme could penetrate into the loose crystallites and produced short chain segments as reflected by the increase in tight β -turn fractions (Fig. 8). We hypothesise that the tight turns and other short segments, due to their increased mobility, could form strong aggregates through hydrogen bonding and/or cross linking. Less amorphous domains and the presence of these strong aggregates increased the degradation temperature of enzyme treated powder. WAXS result as discussed already also supports formation of such aggregates with a short range order.

Discussion

About a three-fold increase in the degradation rate in silk powders compared to silk fibres is evident in Fig.2. Milling of fibres produces ultrafine porous particles. Surface area of average 5 μm particles is equivalent to 200-300 nm solid spheres due to their highly porous morphology [28]. Surface area is an important contributor to the rate and extent of enzymatic degradation of silk fibroin as the mass loss happens through a surface erosion process [20]. The first step of degradation is surface-binding and the second step is enzymatic hydrolysis of the protein backbone[29]. Hence both fineness and porous architecture contribute to the scale and kinetics of degradation. Enzyme treatment is also responsible for a substantial reduction in random coil content which is likely to increase brittleness. Fragmentation of such brittle porous powders results in ultrafine particles as reflected in Fig.3 and Fig.4.

Fig.11 provides a model of the microstructure changes in silk fibroin due to milling and enzymatic hydrolysis. Crystals in silk are formed by stacking of β -sheets which in turn are formed by hydrophobic segments of silk fibroin heavy chains [30, 31]. Hydrogen bonds are responsible for intermolecular forces in the β -sheets. Relatively weak van der Waals interactions provide the stacking intermolecular forces between the sheets. On milling, the mechanical energy is used to fragment the fibres. The milling energy breaks the weak van

der Walls forces disturbing the regular stacking order of the β -sheets. The change in silk fibroin structure after milling is reflected in WAXS peak position and intensity (Fig.9) and reduction in degradation temperature and increase in $T_g(1)$ (Fig.10). However, FTIR studies suggest that the intramolecular hydrogen bonds within the β -sheets are retained in the particles providing structural stability and hence silk powders remain insoluble. We earlier reported adequate stability of the particles with some partial solubility in hexafluoroisopropanol (HFIP), which could be advantageously used to develop porous silk-silk composite scaffolds [8, 9, 32]. The stability against quick dissolution of the particles can be attributed to adequate intermolecular β -sheet forces while partial solubility can be attributed to the reduced intermolecular β -sheet after milling. An open structure provides enzyme molecules better access to the silk micro structure. Such insight is important for design of particles for optimum processing and control of mechanical and degradation properties of particle based systems.

The mechanism of particle degradation is also shown in Fig.11. Enzyme predominantly acts on the hydrophilic segments reducing random coil proportion in the structure (Fig.8). Relatively compact β -sheets largely remain resistant to hydrolysis, but rupture of random coils linking the β -sheets leads to a progressive removal of sheets from the structure (dotted circles in Fig.11). The reduction in β -sheet crystallite size during enzyme treatment causes progressive drop in WAXS peak intensity with the increase in treatment time. The process of separation of β -sheet and random coils creates holes in the structure. As the hydrolysis continues, large holes are created in the particle surface as evident in highly magnified SEM images in Fig.11 leading to surface erosion. According to this mechanism, depending on the rupture sites, the degradation fragments at any particular time can vary in size. An earlier analysis of protease XIV degradation suggested formation of a combination of small (less than 50KD) and large (more than 200 KD) segments [33], which supports the model. Solubilized fractions removed from the particle structure are removed when enzyme solution is replenished with a fresh solution. The supernatant was clear without any turbidity indicating that there were no large insoluble fragments in the solution and mass loss was due to separation of small solubilized fragments only. FTIR results of residual mass at the early part of the enzyme treatment show only marginal change in the secondary structure despite significant loss of mass. Thus both random coil and β -sheet fragments are dissolved and removed. The large fragments of freed segments remain insoluble. Earlier studies suggest that protease XIV can degrade soft segments within the β -sheets [11]. We therefore propose that as the process continues the large insoluble β -sheets freed from the particles are accessed by enzyme molecules leading to their hydrolysis and generation of fragmented sheets and small β -turns and bands. Depending on the nature, size and conformation of β -sheet and their fragments, they are either dissolved or due to their increased mobility, form aggregates (tight β -turns) through hydrophobic interactions or forms cross links. Similar cross linking of short chain segments through $-NH_2$ and $-OH$ end groups due to chain breakage and associated increase in modulus of silk fibres during thermal treatment have been reported[25]. Proportional increase in tight β -turn fractions at a later part of degradation is confirmed by FTIR results (Fig.8). Chen et al. provided evidence that turns are formed easily from shorter mobile chain fragments and cause increase in peak at 1693 cm^{-1} [23]. The aggregates propose in this model increase FTIR peak intensity around 1697 cm^{-1} causing a proportional increase of tight β -turn fraction. They do not contribute to WAXS peak but together with reduced random coil fraction is responsible for higher thermal degradation temperature of enzyme treated powder.

Thus about a three fold increase in mass loss in silk particles compared to silk fibres can be attributed to higher surface area, porous structure of milled particles and an open microstructure due to less compact β -sheet crystals. The reduction in flexible hydrophilic

amorphous domains leads to increased brittleness helping fracture of porous particles into finer fragments.

Conclusions

Mechanical energy in milling opens up the silk microstructure by overcoming weak intermolecular hydrophobic forces between the stacked β -sheets within the silk crystallites. The open microstructure results in higher $T_g(1)$ and lower degradation temperature. High surface area, porous morphology and reduced crystallinity of silk particles are responsible for nearly a three-fold increase in mass loss during enzymatic hydrolysis compared to fibres. Volume based $d(0.5)$ of particles reduced from 6.4 μm to 1.8 μm within the experimental biodegradation time which is attributed to fracture of porous and brittle particles. Experimental data on morphology and structural of fibres and particles can be used to design a model of microstructural change during milling and biodegradation. The model demonstrates enzyme action in the hydrophilic links between the crystallites progressively removing the β -sheets. Degraded particles show a higher resistance to thermal degradation and sharper peak due to reduced proportion of amorphous domain and formation of tight aggregates by β -sheet fragments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig.1.

Volume median particle size (d 0.5) of silk powder at different time of rapid degradation tests; 0: at the start; 30 min: 30 min degraded powder; 4 h: 4 hour enzyme degraded powder; 1 d: 1 day enzyme degraded powder; 3 d: 3 days enzyme degraded powder; 6 d: 6 days enzyme degraded powder; 12 d: 12 days enzyme degraded powder; 12 d (PBS): 12 days PBS only treated powder; (Protease XIV 2mg/mL);

Fig 2.
Change of mass of fibre and powder during enzyme degradation tests: slow tests (1mg/mL protease XIV) and rapid degradation tests (2 mg/mL protease XIV)

Fig.3.
Particle size distribution at various time of rapid degradation test.

Fig.4.
SEM images of silk powder during degradation (Protease XIV: 1mg/mL).

Fig.5. SEM images of silk fibres: a) Fibre before degradation; b) fracture of a degraded fibre in 12 d; c) surface erosion of a degraded fibre in 12 d.

Fig.6.

FTIR Amide I region (F0: Degummed fibre at start; P 12 d(B): 12 days PBS only treated powder; P 12 d: 12 days enzyme degraded powder; P 30min: 30 minutes enzyme degraded powder); Protease XIV: 2 mg/mL.

Fig.7. Curve fitted FSD spectra of amide I regions (B-ter: inter molecular β -sheet, B-tra: intramolecular β -sheet, r: random coil, t: β -turn, tt: tight β -turns; (Protease XIV: 2mg/mL).

Fig.8.

Percentage of different conformations; F0: Degummed fibre at the start; P 12 PBS: Day 12 PBS only treated powder; P 30 min: 30 min enzyme degraded powder; P 3 d: 3 days enzyme degraded powder; P 12 d: 12 days enzyme degraded powder; data are from rapid degradation studies (Protease XIV: 2mg/mL).

Fig.9. Wide angle X-ray diffraction spectra (F: Fibre; P 12 d(PBS): 12 days PBS only treated powder; P 12 d: 12 days enzyme degraded powder; (Protease XIV: 2mg/mL).

Fig.10. Differential scanning calorimetry curves (F: Fibre; P 12 d(B): 12 days PBS only treated powder; P 3 d: 3 days enzyme degraded powder; P 12 d: 12 days enzyme degraded powder; (Protease XIV:2 mg/mL).

Fig.11.
Schematic diagram and SEM images of the degradation mechanism.